

ENZYME ACTIVITIES OF MAIZE (Zea mays L.) ENDOSPERM
CULTURES FOLLOWING SUCROSE SUPPLEMENTATION

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN HORTICULTURE

AUGUST 1974

By

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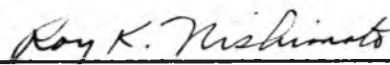
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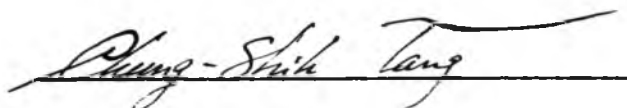
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ACKNOWLEDGMENTS

The author wishes to express her sincere appreciation to the people and government of the United States of America for their assistance that enabled the author to study in this country. Special thanks are extended to Dr. J.C. Shannon for his providing the maize endosperm tissue cultures for this study and valuable guidance and assistance while the author was at The Pennsylvania State University.

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INTRODUCTION

Detailed pathways have been suggested for sucrose degradation and glucose transfer in the formation of starch in plants on the basis of cell-free systems. However, the actual mechanisms active in plant cells can only be determined unequivocally through studies of the intact system. Due to certain limitations associated with the study of intact plants, maize tissue cultures were used in an attempt to overcome these limitations. The formation of starch in the cultured cell was shown to increase with sucrose supplementation to the culture medium. Several enzymes which are involved in sucrose degradation (invertase, sucrose-UDP glucosyltransferase), starch formation (phosphorylase, starch synthase), and starch degradation (amylase) were studied to determine their activities in the cultured endosperm cells following sucrose supplementation.

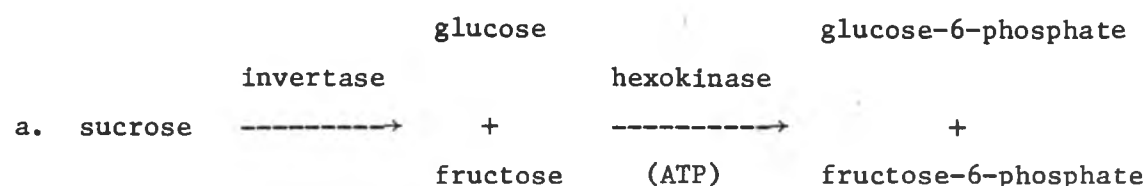
Peroxidase isozyme studies were conducted as a means of evaluating similarities between the endosperm tissue cultures and the developing endosperm. In several plant species, peroxidases have been shown to be tissue- or organ-specific. Variations of peroxidase isozymes have also been reported at different developmental stages. Peroxidase isozyme polymorphism in both developing endosperm and endosperm tissue cultures were studied and ontogenetic variations of peroxidase isozymes in these two tissues were determined.

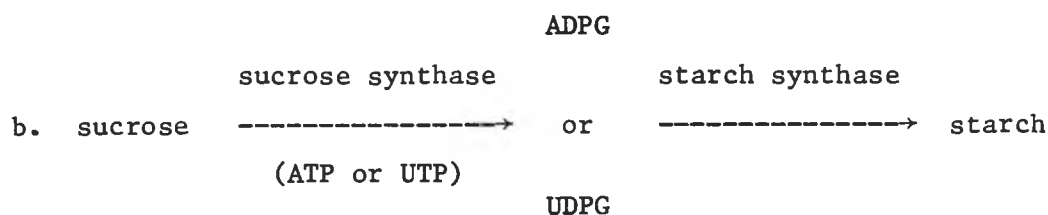
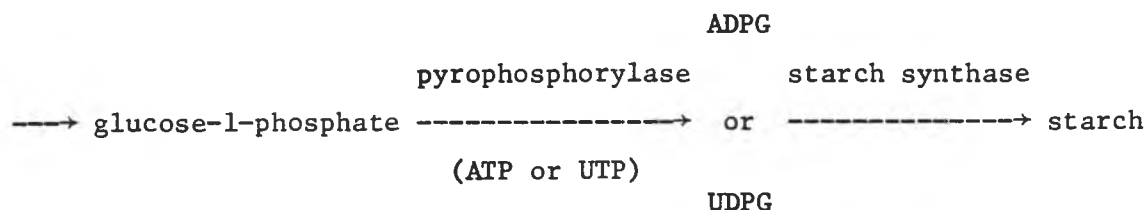
LITERATURE REVIEW

Enzymes which are involved in sucrose degradation (invertase, sucrose-UDP glucosyltransferase), starch formation (phosphorylase, starch synthase) and starch degradation (amylase)

Starch is a major reserve compound in plants, found primarily in storage tissues such as the kernels of cereal plant, and the tubers and roots of many species. Photosynthetically formed sugars are immediately converted to starch in chloroplasts; such starch is called "assimilation starch". Assimilation starch is a transient form that is utilized by metabolic processes or transformed into storage or "reserve starch" in organs such as fruits, seeds, stems and roots (Akazawa, 1965). While starch is scarcely detectable in grass leaves, sucrose is present in large amounts and is the main transport carbohydrate (Porter, 1962). Sucrose may be converted into reserve compounds in specific organs. Starch is synthesized in the endosperm cells of cereal crops as a reserve polysaccharide, to be used as an energy source during germination (Akazawa, 1965).

The mechanism of sucrose-starch interconversion has been studied in sweet corn (De Fekete and Cardini, 1964) and rice (Akazawa et al., 1964). Akazawa (1965) concluded that there are two possible pathways of sucrose-starch interconversion:





Invertase (β -fructofuranosidase)

The essential part of the pathway (a) above involves β -fructofuranosidase (β -D-fructofuranoside fructohydrolase E.C. 3.2.1.26) for the breakdown of sucrose. This enzyme catalyzes the hydrolysis of the non-reducing β -D-fructofuranoside residue in sucrose and this results in the loss of 6,600 cal / mole, the free energy of hydrolysis by the glycosidic bond at pH 7.0 (Akazawa, 1965).

Enzymes capable of catalyzing sucrose hydrolysis occur in several conditions in plant tissues. β -Fructofuranosidase was first extracted from yeast in 1860 by Berthelot, who referred to it as invertase. Since 1950, many reports on the occurrence of invertase in plant organs have appeared (Myrbäck, 1960). A cell wall-bound invertase was found in yeast (Hoshino et al., 1964) as well as in higher plants (Kivilaan et al., 1961). Bound invertase was associated with the cell walls and could not be removed by treatment with salt solution or organic solvents (Hawker and Hatch, 1965). In addition to the distinction of bound and soluble forms, multiple forms of the soluble invertase were found. For example,

immature tissue of sugar cane had an acid invertase (optimum pH 5.0 - 5.5) whereas mature tissue had a neutral invertase (optimum pH 7.0) (Hatch et al., 1963). Similarly, Jaynes and Nelson (1971) reported that there were two soluble invertases and a bound invertase in the developing endosperm of maize. However, the relationship between the different forms of invertase and sucrose metabolism is unclear.

Sucrose-UDP glucosyltransferase (sucrose synthase)

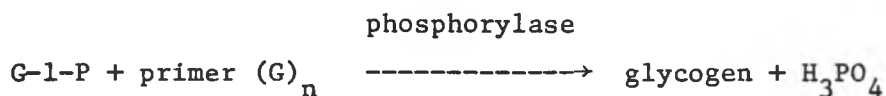
The initial step of the second pathway (b) involves sucrose synthase (UDP-glucose : D-fructose 2-glucosyltransferase E.C. 2.4.1.13). This enzyme was first found by Cardini, Leloir and Chiriboga (1955) in wheat germ. It utilizes D-fructose as the D-glucose acceptor for sucrose formation and performs an important function in the degradation of this disaccharide (Nikaido and Hassid, 1971). They postulated that sucrose may be completely degraded by this enzyme if the UDP-D-glucose is exhausted and UDP is present in excess. De Fekete and Cardini (1964) found that UDP is the preferred substrate among the nucleotide diphosphates for this glucosyltransferase. They suggested that UDP-glucose may have a primary role in the mechanism of the sucrose-starch transformation.

Phosphorylase

Glucose may be added to the primer either from glucose-1-phosphate by the action of phosphorylase, or from nucleoside diphosphate glucose by the action of starch synthase. The glucose-1-phosphate substrate can be produced from the phosphorylation of free hexose catalyzed by

hexokinase and phosphoglucomutase (Akazawa, 1965). The nucleoside diphosphoglucose substrates can be directly produced from sucrose as discussed above or from glucose-1-phosphate and nucleoside triphosphate via the action of pyrophosphorylase (Espada, 1962).

Phosphorylase (α -1,4 D-glucan : orthophosphate α -glucosyltransferase E.C. 2.4.1.1) was first found in liver tissue where it catalyzes a reversible reaction between glucose-1-phosphate (G-1-P) and glycogen (Cori et al., 1937).



In this reaction, the energy level of the glucosidic linkage ($\Delta F^\circ = -4300$ cal / mole) in the polysaccharide molecule is approximately the same as that of the phosphate ester linkage of the Cori-ester ($\Delta F^\circ = -4800$ cal / mole). Thus, the overall reaction is reversible. Soon after this discovery, the same type of reaction was demonstrated in tissues of higher plants (Hanes, 1940). It was then assumed that phosphorylase might be playing an important role in glycogen and starch biosynthesis and degradation. However, animal and plant enzymes have different primer requirements (Whelan and Bailey, 1954).

Phosphorylase was once considered only as a degradative enzyme. The equilibrium of the phosphorolysis reaction was found to be pH-dependent (Hanes and Maskell, 1942). Low pH values and low Pi/G-1-P ratios were found to favor starch synthesis. However, from the studies of the pH value and the equilibrium in some starch-containing plant tissues, Ewart et al. (1954) and Rowan and Turner (1957) found that higher pH values and high Pi/G-1-P ratios were present at the sites of

starch synthesis. They postulated that phosphorylase is involved only in starch breakdown, rather than in starch synthesis. The discoveries of UDP-glucose glycogen glucosyltransferase in liver tissue (Leloir and Cardini, 1957), ADP-glucose starch glucosyltransferase in higher plants (De Fekete et al., 1960), and evidences of starch synthesis predominantly via glucosyltransferase (to be discussed later) led Nordin and Kirkwood (1965) to state:

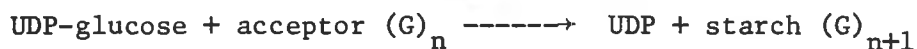
"There now can be very little doubt that the synthesis of amylose, amylopectin and phytoglycogen all proceed from nucleoside diphosphate glucose intermediates, and that the older notion that the primary synthesis involves the action of phosphorylase on G-1-P must be totally abandoned and phosphorylase regarded as being concerned only with the degradation of starch."

However, there are other studies which have suggested that phosphorylase is important in the synthesis of starch. Illingworth and co-workers showed that highly purified phosphorylase of muscles and potatoes is capable of forming an amylose chain from G-1-P in the absence of a primer (Illingworth et al., 1961). This suggested that phosphorylase could be of special importance in the early stage of polysaccharide biosynthesis. Badenhuizen (1963) also reviewed that phosphorylase may be one of the primary enzymes in starch synthesis. Recently, Tsai and Nelson (1968, 1969) isolated three different phosphorylases (phosphorylase I, II and III) from developing corn kernels. The activity of phosphorylase II and phosphorylase III increased during the period of most active starch synthesis and deposition, and were absent from germinating kernels. Phosphorylase II was active in synthesizing an amylose-like polymer from a "primer free" system. They concluded that enzymes II and III were involved in starch synthesis. In addition, Slabnik and Frydman (1970) isolated a

phosphorylase from potato tubers which catalyzed the formation of an amylopectin-like polysaccharide in a cell-free system without the addition of primer. They suggested that this particular phosphorylase may function in starch synthesis in this particular potato.

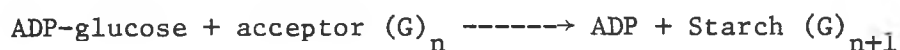
Nucleoside diphosphate-glucose : starch glucosyltransferase (starch synthase)

Uridine diphosphate-glucose : glycogen 4- α -glucosyltransferase (E.C. 2.4.1.1) was first found by Leloir and Cardini (1957) in liver tissue. Later, Leloir and his co-workers (1961) found a similar enzyme associated with starch grains from beans, potatoes and corn seedlings. Thus, they proposed a new mechanism of starch biosynthesis as follows:



De Fekete, Leloir and Cardini (1960) pointed out that the acceptor may be starch or a di- or oligo-saccharide of the maltose series. The type of glucosidic linkage synthesized was $\alpha(1 \rightarrow 4)$. The free energy change of this reaction was -3300 cal which favored the synthetic reaction. The effective synthesizing enzyme was found to be bound to starch granules and could not be dissociated from them.

Adenosine diphosphate-D-glucose (ADPG) was also found to be a substrate of this starch biosynthetic reaction. Recondo and Leloir (1961) found that ADPG reacted ten times more rapidly than UDPG in bean starch synthesis, and proposed that the starch biosynthetic mechanism involved ADPG as follows:



Initially, a synthetic ADPG was used, but later this nucleotide was found to be a natural constituent in maize (Recondo et al., 1963) and rice (Murata et al., 1963).

A soluble enzyme preparation was obtained from sweet corn that catalyzed the transfer of the D-glucosyl group from ADPG to phytoglycogen, which has a branched chemical structure similar to that of glycogen (Frydman and Cardini, 1965). The soluble glucan synthase was also capable of utilizing dADP-D-glucose, although to a lesser extent. The same investigators extracted this soluble enzyme from a number of different plants (Frydman et al., 1966). The soluble glucosyltransferases obtained from different plant sources required different glucosyl acceptors. Ozbun et al. (1971) separated two soluble ADPG : starch glucosyltransferases from immature waxy maize. Both enzymes transferred glucose from ADPG to amylopectin, amylose, glycogen, maltotriose and maltose as primers but there were marked differences between their ability of glucose transfer to amylopectin and glycogen. One of these two enzymes was found to produce a starch-like polysaccharide without a primer.

Tanaka and Akazawa (1968) concluded that the bound and soluble starch synthase are different enzymes. Akatsuka and Nelson (1969) also made a similar conclusion based upon other reasons. Nikaido and Hassid (1971) stated in a review paper that the different primer requirement between the bound and soluble starch synthase might indicate that they are different enzymes, or that both activities are due to the same enzyme in different conformations.

Nucleoside diphosphate-glucose pyrophosphorylase

ADP-glucose pyrophosphorylase (ATP : α -D-glucose-1-phosphate adenyl transferase E.C. 2.7.7.27) was first found in wheat flour (Espada, 1962). It catalyzed the following reaction:



The existence of this enzyme in plants further supports the hypothesis that the formation of starch can proceed from nucleoside diphosphate-glucose.

Tsai and Nelson (1966) reported that in starch-deficient maize mutants shrunk-2 and brittle-2, ADP-glucose pyrophosphorylase activity was void in both endosperm and embryo tissue. These two mutant kernels accumulated 25 % as much starch as the normal ones. Therefore, Tsai and Nelson suggested that the major pathway of starch synthesis occurred via the ADP-glucose pyrophosphorylase and ADP-glucose : starch glucosyltransferase reactions. In subsequent studies by Dickinson and Preiss (1969) results definitely indicated the presence of low but significant levels of ADP-glucose pyrophosphorylase in the endosperm of shrunk-2 and brittle-2 maize kernels (12 % and 17 % of the normal ones, respectively). These data still support the idea that the basis for the low amounts of starch in the endosperm of these maize mutants is the low activity of ADP-glucose pyrophosphorylase.

Nucleotide diphosphates are therefore very important in starch synthesis. Both phosphorylase and nucleoside diphosphate-glucose glucosyltransferase build the $\alpha(1 \rightarrow 4)$ glucosidic linkage, and other branching enzymes are required to produce amylopectin (Akazawa, 1965).

Amylase

The term amylase has been used to designate the enzymes that catalyze the hydrolysis of $\alpha(1 \rightarrow 4)$ glucosidic linkages of polysaccharides such as starch and glycogen (Fischer and Stein, 1960). They have been subdivided into two groups, α -amylase and β -amylase, according to their modes of action.

Alpha-amylase, which is more precisely called 1,4- α -D-glucan glucanohydrolase (E.C. 3.2.1.1), was crystallized from germinating barley endosperm by Schwimmer and Balls (1949). It attacks both amylose and amylopectin randomly throughout the molecule. Ca^{++} is required for this enzyme activation and also for stabilization against proteolytic destruction and thermal denaturation (Akazawa, 1965).

Beta-amylase, or more precisely 1,4- α -D-glucan maltohydrolase (E.C. 3.2.1.2), has been isolated in crystalline form from various plants, such as sweet potato (Balls et al., 1946) and germinating barley seed (Fischer et al., 1950). It cleaves away successive maltose units beginning from the nonreducing end of amylose or amylopectin. However, β -amylase cannot pass the branch points, $\alpha(1 \rightarrow 6)$ linkages. Therefore, amylopectin will only be partially hydrolyzed, yielding the residual β -limit dextrin. Beta-limit dextrin, which is not further susceptible to β -amylase, can however be hydrolyzed by α -amylase in the inner core portion, leaving the branch point intact.

Multiple forms of amylases have also been reported in barley (Frydenberg and Nielsin, 1965), tobacco crown-gall tissue culture (Jaspars and Veldstra, 1965), maize (Scandalios, 1966) and rice (Tanaka et al., 1970). However, the biological function of each enzyme still

remains obscure. There is no maltose or dextrin present in plant cells as natural constituents; thus, it is obvious that amylases are not involved in starch synthesis but only in starch degradation (Akazawa, 1965).

The use of endosperm cultures to study starch biosynthesis

Gautheret (1955) discussed the use of tissue cultures for nutritional and metabolic studies. Plant tissue and organ cultures have been used in studies of the biosynthesis of alkaloids (Dawson, 1960), anthocyanins (Straus, 1959), cell wall components (Lamport and Northcote, 1960) and phenolic compounds and lignin (Li et al., 1970). The feasibility of using plant tissue culture for the study of starch biosynthesis was revealed by Batey (1971).

Detailed pathways of sucrose hydrolysis, glucose transfer and starch formation are proposed on the basis of information obtained from cell free, isolated enzyme studies. However, in vivo studies should be conducted to determine the mechanism of active enzymatic pathways in the intact cell. In an attempt to overcome some of the limitations associated with intact plants, the plant tissue culture system was used for in vivo studies. Shannon (1973) has discussed several advantages of using corn endosperm tissue cultures for in vivo starch synthesis studies. One advantage of this system is that a continuous supply of tissue is available. Second, it is an in vivo system, since intact metabolically active cells are used. Third, the cultures are composed of single cells and small cell clumps which are ideally suited for pulse radioactive labeling studies. Finally, the capability of "turning-on"

starch synthesis and deposition at a given time allows one to investigate the cytological, ultrastructural, and biochemical events associated with the induction or initiation of starch synthesis and deposition.

Batey (1971) found that actively growing cultures accumulated negligible amounts of starch; however, an upward shift in the sucrose content of the culture medium stimulated starch biosynthesis and deposition. There was an eight- to twelve-day lag period between the addition of supplemental sugar and the accumulation of starch. Changes in nuclear, nucleolar, and plastid morphology during the initiation of starch synthesis, i.e. during the lag period, were similar to those reported to occur in normally developing corn kernels (Lampe, 1931).

Uniform and reproducible growth and starch deposition was obtained from cultures derived from A636, a normal maize inbred (Shannon and Batey, 1973). Induction of starch synthesis and deposition in this culture was studied by the supplementation with different sugars in the culture medium (Shannon, 1973). Sucrose, glucose, and fructose were found to be more effective than galactose. It was also found that a second addition of sucrose induced more starch synthesis and deposition in the cultures than a single sucrose addition.

Maize peroxidase polymorphisms and their ontogeny and tissue specificities

Peroxidase (donor : H_2O_2 oxidoreductase; E.C. 1.11.1.7) isozymes have been detected in a wide variety of animals, plants, and microorganisms. Several techniques have been used to isolate and distinguish them, such as electrophoresis, chromatography, gel filtration,

immunochemistry and sedimentation (L. Shannon, 1968).

In several plant species, peroxidases have been shown to be tissue- or organ-specific. Scandalios (1964) through use of starch gel electrophoresis, demonstrated that cathodic peroxidases in maize showed a large variability between tissues. Hamill and Brewbaker (1969) further concluded that maize tissues varied greatly in peroxidase isozyme patterns, many tissues having a characteristic and distinguishing complement. Recently, levels of activity of twelve peroxidases were established for twenty-one tissues of maize (Brewbaker and Hasegawa, 1974).

In addition to the tissue specificity of peroxidase, peroxidases from different stages in development of the maize sporophyte show distinct variations. The most common phenomena are shifts in enzymatic activity, i.e., progressive increase or decrease in activity (Scandalios, 1969). Hamill and Brewbaker (1969) also studied the ontogenetic variations of the leaf blade, leaf sheath, and internode during maturation. They found that definite variations were related to the rates of tissue enlargement.

Some peroxidases were found to be cell wall-bound. Ca^{++} has been used to release peroxidase from the wall-bound fraction (Lipetz and Garro, 1965). Brewbaker and Hasegawa (1974) further reported that all cathodal enzymes (isozymes 1, 4, and 5) were associated with the cell wall. However, the significance of cell wall-bound, tissue-specific peroxidases is still not clear.

MATERIALS AND METHODS

A. Preparation of plant materials

Endosperm cultures from the field corn inbred A636 of maize (Zea mays L.) were established in 1970 (Shannon and Batey, 1973). The endosperm cultures used in this experiment were obtained from Dr. J.C. Shannon, which were subcultured from a "mother culture". After subculturing, starch synthesis in the endosperm cultures was induced by the addition of six grams of sucrose to the culture medium. Subsequently, endosperm cultures were harvested 0, 8, 16, and 24 days after sucrose supplementations. The cultured tissue was freeze-dried and stored at -20 C until processing.

Developing kernels of the same inbred A636 of maize were collected 10, 14, and 18 days after controlled self-pollination at the Waimanalo Farm in Hawaii. They were stored at -20 C until processing.

B. Enzyme preparation

Each freeze-dried endosperm culture was homogenized and extracted with 0.01 M Tris-maleate buffer (pH 7.0) according to the procedure of Tsai et al. (1970). After centrifugation at 10,000 g for 20 min, the residue was washed twice with the same buffer by suspension and recentrifuged. Part of the combined supernatants was dialyzed overnight at 0 to 4 C against Tris-maleate buffer. This dialyzed extract was used as a crude enzyme for the assay of amylase, soluble invertase, and sucrose-UDP glucosyltransferase activities. The remainder of the crude extract, without dialysis, was used for the phosphorylase assay. The

washed cellular residue was suspended in the above buffer and used as a source of cell wall-bound invertase.

Soluble and cell wall-bound peroxidases were separated according to Hasegawa (1974). Each freeze-dried endosperm culture or frozen endosperm was homogenized with cold distilled water (4 C). The homogenate was centrifuged at 10,000 g for 10 min. The supernatant was used as a source of soluble peroxidases. The residue was washed three times with cold distilled water by suspension and recentrifugation. The washed cellular residue was finally suspended in 0.2 M CaCl_2 at 0 to 4 C for 60 min to release cell wall-bound peroxidases. After centrifugation at 10,000 g for 10 min, the supernatant was decanted and used as cell wall-bound peroxidases preparation.

C. Enzyme assay

Phosphorylase

Phosphorylase activity was measured by the incorporation of radioactivity from uniformly-labeled glucose- ^{14}C -1-phosphate into an amylopectin acceptor following Tsai et al. (1970). In addition, NaF and Hg_2Cl_2 were added to suppress the activities of acid phosphatase and β -amylase (Whelan, 1955; Alexander, 1973).

The reaction mixture for phosphorylase assay contained 11.25 μmoles of glucose- ^{14}C -1-phosphate (30,000 to 35,000 cpm), 10 μmoles of NaF, 1×10^{-4} μmoles of Hg_2Cl_2 , 10 μmoles of MES buffer (2-(N-Morpholine) ethanesulfonic acid) at pH 5.8, and 1 mg of amylopectin in a total volume of 0.315 ml. Amylopectin was solubilized in MES buffer solution by heating in a hot water bath (80 C) before use. The reaction mixture

was incubated at 37 C for 30 min and terminated by the addition of 0.5 ml of 0.1 N NaOH. Subsequently, the amylopectin was precipitated out by the addition of methanol in an ice bath until a final concentration of 75 % methanol was obtained. The pellet was collected by centrifugation (0 to 4 C) and washed three times with 0.1 N NaOH, followed by methanol precipitation in an ice bath and recentrifugation. After resuspension in 0.1 N NaOH, the material was transferred to a 1 x 3 cm strip of glass fiber paper (Whatman GF-82). This strip was dried under heat lamps, then placed in counting vial containing 20 ml of toluene-based scintillation fluid (4 g of Omnifluor [New England Nuclear, Boston, Mass.] per liter of toluene). Radioactivity was determined by using a Nuclear-Chicago ISO CAP/300 scintillation counter.

Sucrose-UDP glucosyltransferase

Sucrose-UDP glucosyltransferase was determined by measuring the release of reducing sugars from sucrose according to Shannon and Dougherty (1972). To correct for invertase activity, the amount of reducing sugars released in the absence of UDP was subtracted from that released in its presence.

The reaction mixture contained 60 μ moles of MES buffer (pH 6.5), 20 μ moles of sucrose, 0.5 μ mole of UDP and enzyme in a final volume of 0.5 ml. After incubation at 30 C for 30 min, the reaction was terminated by the addition of the copper reagent of Nelson's arsenomolybdate method and the reducing sugars released from the sucrose substrate was measured (Hodge and Hofreiter, 1962).

Invertase

Both soluble and cell wall-bound invertase were assayed according to methods of Shannon and Dougherty (1972). The reaction mixtures contained 40 μ moles of sodium acetate buffer (pH 4.8), 5 μ moles of sucrose and enzyme in a total volume of 0.4 ml. The reaction mixture was incubated at 37 C for 30 min; the reaction was terminated by the addition of the copper reagent of Nelson's arsenomolybdate method. The reducing sugar produced was measured (Hodge and Hofreiter, 1962). The free sugar in the crude enzyme extract was corrected with the zero time as control.

Amylase

The method of Chrispeels and Varner (1967) was used for the assay of amylase activity. This method was based on the loss of OD_{620} absorption of a soluble starch-iodine complex. 0.5 ml of freshly-prepared 0.15 % potato starch solution containing 0.6 % KH_2PO_4 and 0.01 M $CaCl_2$ was incubated with 0.1 ml enzyme extract at 30 C for 10 min. The reaction was stopped by the addition of 1 ml of acidic iodine reagent. The iodine reagent was prepared by dissolving 6 gm KI and 0.6 gm I_2 in 100 ml H_2O and diluted with 0.05 N HCl (1 : 100) before used. The difference in optical density between zero time and that taken after incubation was regarded as the activity of amylase.

Peroxidase

Horizontal gel electrophoresis for both soluble and cell wall-bound peroxidases were conducted according to the methods used by Brewbaker

et al. (1968). Gels of 7 % polyacrylamide were electrophoresced using lithium-borate buffer (pH 8.1) at 6-8 v/cm for 10 to 12 hrs at 4 C. Benzidine dihydrochloride was used as a hydrogen donor for staining gels.

Soluble protein

Soluble protein was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as standard. Each enzyme assay was conducted three times. The entire experiment was repeated. The data are presented as the average of the two experiments.

RESULTS AND DISCUSSION

Starch content of maize endosperm cultures following sucrose supplementation

The enzymatic studies to be reported were of maize endosperm tissue cultures that had been primed with sucrose as a stimulus to starch synthesis. Dry weights and starch contents of these cultures increased in the 24-day period after sucrose supplementation (Table 1)¹. The greatest increase in dry weight and starch content occurred during the first 8 days. Dry weight increased approximately 2-fold while starch content increased approximately 7-fold, clearly demonstrating that the addition of sucrose to cultured cells acted as a primer for starch synthesis. Continued increases of starch content were at a lower rate. This may have been a result of reduced nutrient content, especially sucrose, or of inhibitors released by the cells.

The starch synthesized in the cultured endosperm cells did not exceed 9 percent of the dry weight at 24 days after sucrose supplementation. Tsai et al. (1970) reported 9.2 percent starch in the endosperm 12 days after pollination in situ, with a rapid increase to 46 percent at 14 days and to a maximum of 77 percent at 22 days. They suggested that the 12-day stage represented a developmental switch point. Beyond this point, there was a considerable increase in the

¹Data in Table 1 were obtained through the courtesy of Dr. J.C. Shannon, Dept. of Horticulture, Pennsylvania State University, under whom the studies were initiated.

Table 1. Dry weight and starch content of the maize endosperm cultures after sucrose supplementation (Shannon, 1973)

| | Days after sucrose supplementation | | | |
|-------------------------------------|------------------------------------|-------|-------|-------|
| | 0 | 8 | 16 | 24 |
| Dry weight (gm) | 0.856 | 1.654 | 2.061 | 2.315 |
| Starch content (gm) | 0.016 | 0.107 | 0.193 | 0.199 |
| Starch content (% of dry weight) | 1.61 | 6.50 | 8.74 | 7.62 |

activity of several enzymes thought to be involved in starch biosynthesis. The endosperm tissue cultures used in these studies were started from explants 10 days after pollination (Shannon and Batey, 1973). It is possible that these explants were taken prematurely and did not contain or were unable to synthesize the cofactors, enzymes or hormones necessary for the developmental switch point.

Enzyme activities of maize endosperm cultures following sucrose supplementation

Freeze-dried tissue cultures were used throughout this study. Preliminary tests showed that the activities of phosphorylase, sucrose-UDP glucosyltransferase, amylase, soluble and cell wall-bound invertase determined in the freeze-dried tissue cultures were not significantly different from frozen cultures.

Invertase

Preliminary tests showed that inhibitors and activators were absent in the reaction mixture, since both soluble and cell wall-bound invertase activity were found to be directly proportional to the enzyme concentration ($r = 0.995$ and 0.989 , respectively). The concentration of soluble invertase varied from 0 to $125.6 \mu\text{g protein / ml}$, while cell wall-bound invertase was varied from 0 to $2.0 \text{ mg dry tissue / ml}$ (Appendix Figures 7 and 8).

Both soluble and cell wall-bound invertase activities were studied in the endosperm cultures following sucrose supplementation. The specific activity of soluble invertase was defined as $\text{n mole glucose / mg}$

protein - min, while cell wall-bound invertase was defined as n mole glucose / mg dry tissue - min. Soluble invertase activity remained high and fairly constant after sucrose supplementation although some decrease was evident (Figure 1). Cell wall-bound invertase activity decreased rapidly in the first eight days and continuously decreased at a somewhat lower rate (Figure 2). The decrease of bound invertase activity seems to have a log function with time (Appendix Figure 9).

The starch content of endosperm cultures increased after sucrose supplementation, and protein percentages varied in dry weight of tissue. Bound invertase thus should have been defined on the basis of protein content, instead of dry weight of tissue as used in this experiment. If cell wall-bound invertase activity was defined as n mole glucose / mg protein - min, a more meaningful comparison might have been made between soluble and bound invertase.

The activity of bound invertase was assayed by using cellular debris suspension in the reaction mixture. The activity was found to be variable among replicates and resulted in large standard deviation values. Similar variations of bound invertase activity were also reported in maize and tobacco tissue cultures (Straus, 1962) and developing maize endosperm (Janes and Nelson, 1971).

Shannon and Dougherty (1972) observed that with increasing maize kernel age, the activity of soluble invertase in the placento-chalazal tissue declined and cell wall-bound invertase activity increased. They suggested that uptake of sucrose in this specific tissue might be regulated by soluble and cell wall-bound invertase. Possible reasons for the discrepancies between Shannon and Dougherty's work and the

Figure 1. Soluble invertase activity of endosperm cultures after sucrose supplementation.

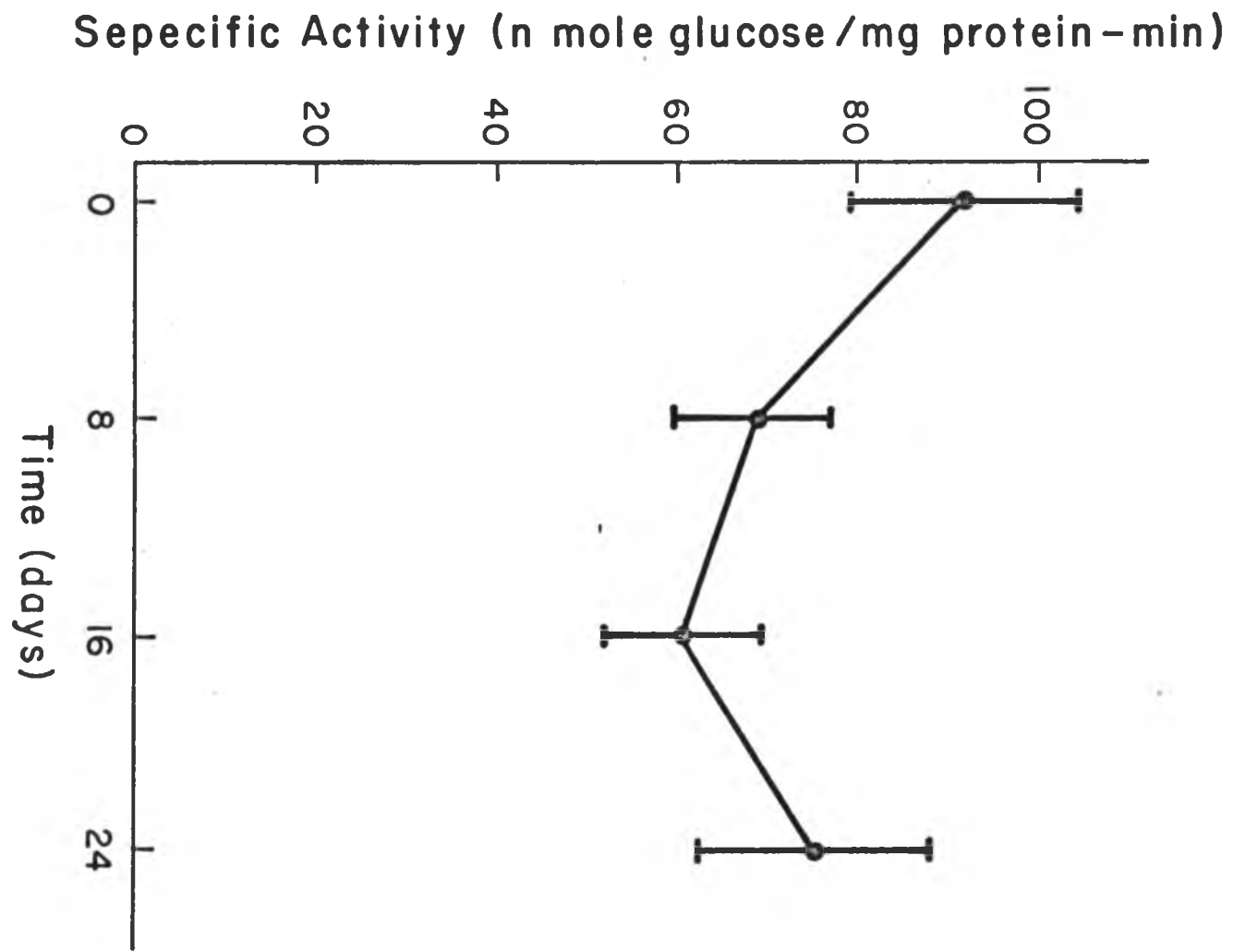
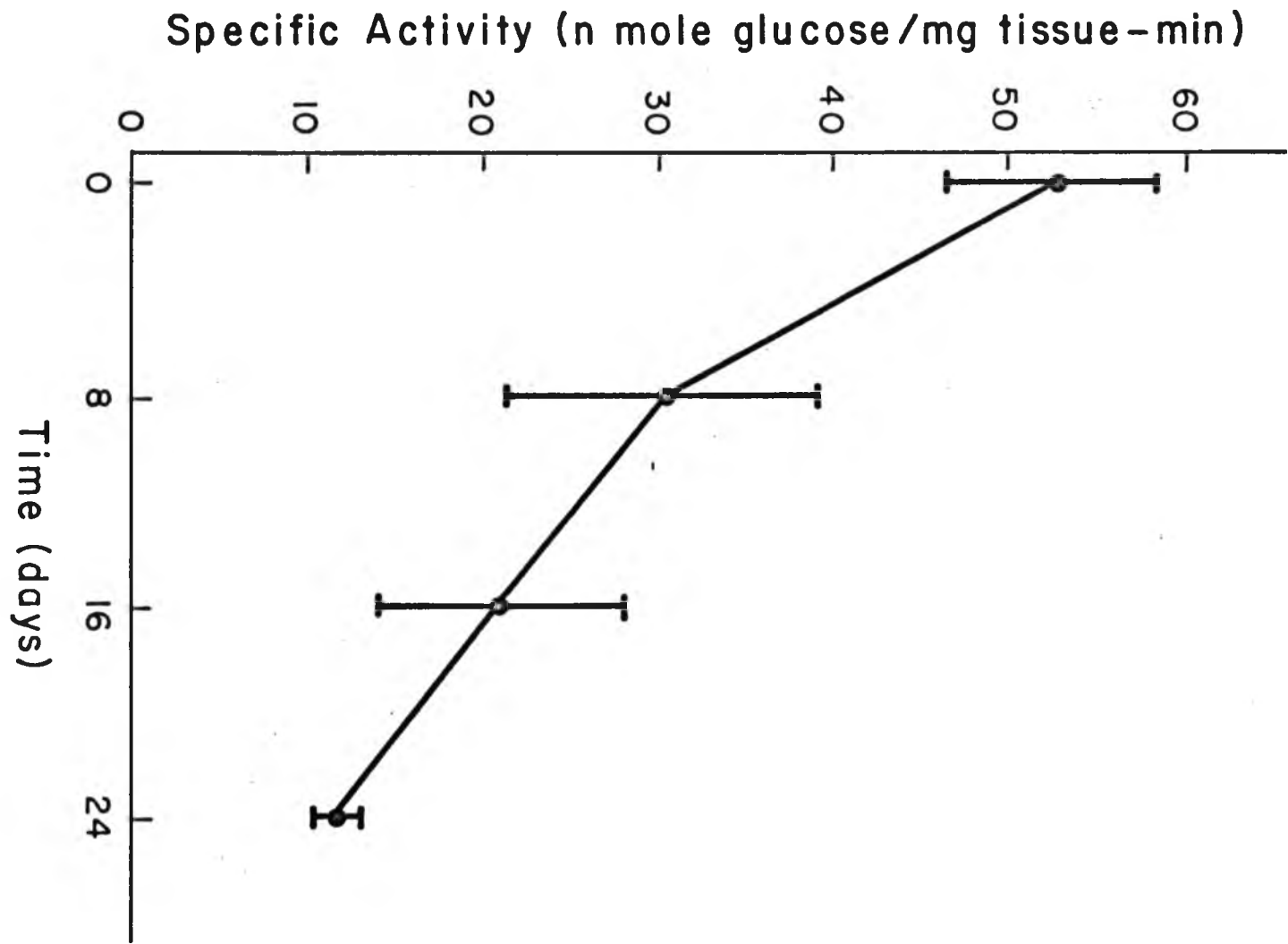


Figure 2. Cell wall-bound invertase activity of endosperm cultures after sucrose supplementation.



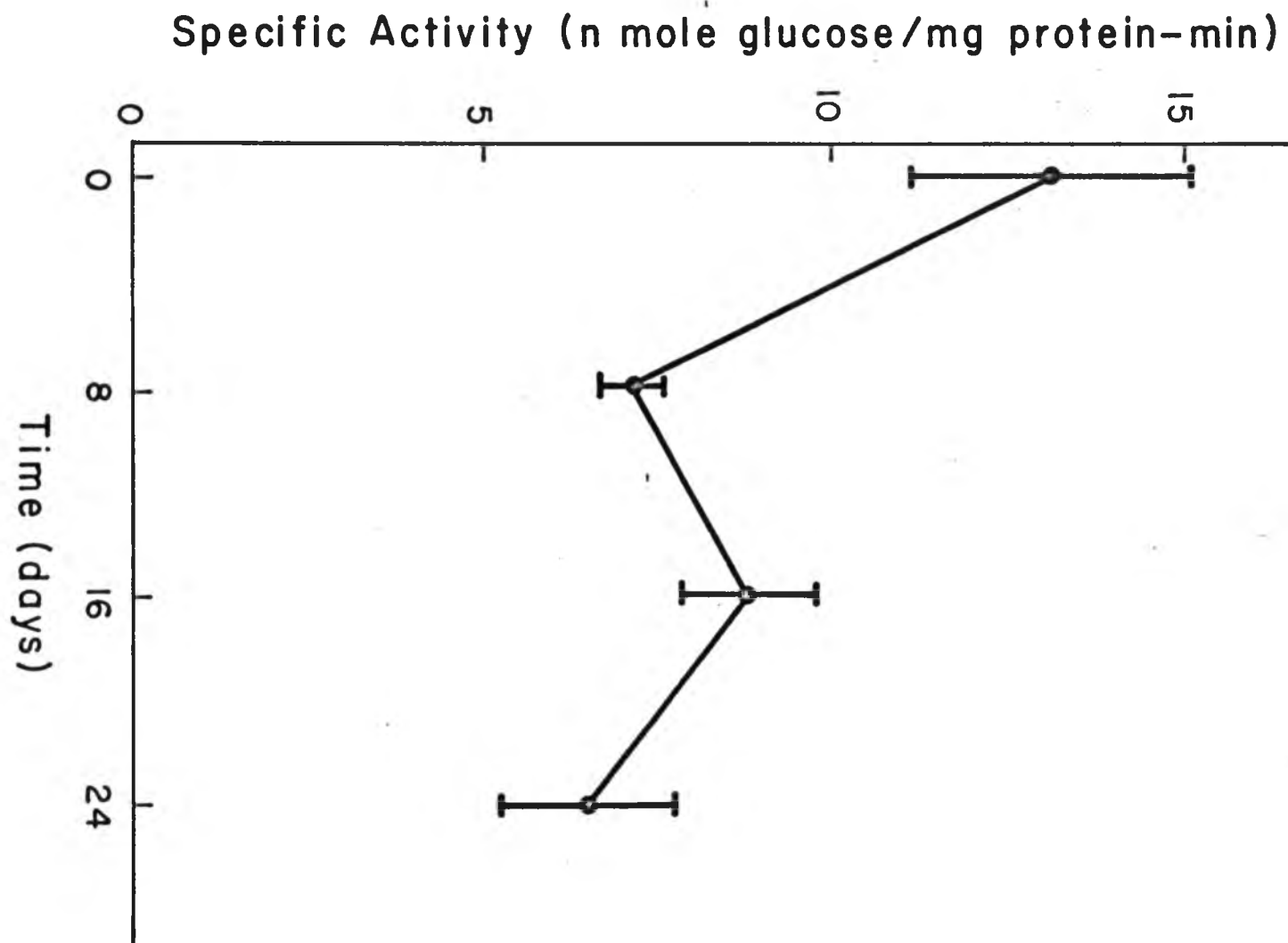
present results may be attributable to the different tissues used.

Sucrose-UDP glucosyltransferase

The specific activity of sucrose-UDP glucosyltransferase varied with time after sucrose supplementation in the endosperm cultures (Figure 3). It decreased markedly in 8 days and remained relatively stable thereafter.

In corn De Fekete and Cardini (1964), and in rice Murata et al. (1966), discussed the physiological role of this enzyme in developing endosperm. They suggested that it catalyzed the first step in the transfer of glucose to a nucleoside diphosphate in the endosperm. Tsai et al. (1970) studied both soluble invertase and sucrose-UDP glucosyltransferase activity in developing corn endosperm. They reported that soluble invertase activity reached its maximum at the 12-day stage and rapidly declined after this period. However, sucrose-UDP glucosyltransferase activity was not detectable until the 12-day stage and its activity increased after starch synthesis had commenced in the developing endosperm. Shannon and Dougherty (1972) found that soluble invertase activity was high in the corn placento-chalazal and pedicel tissue while sucrose-UDP glucosyltransferase was not detectable in these tissues. It is probable that these two sucrose degrading enzymes have different physiological functions in different tissues and during different developmental stages. In this study, invertase activity was much higher than sucrose-UDP glucosyltransferase activity at all stages in the endosperm cultures after sucrose supplementation. Therefore,

Figure 3. Sucrose-UDP glucosyltransferase activity of endosperm cultures after sucrose supplementation.



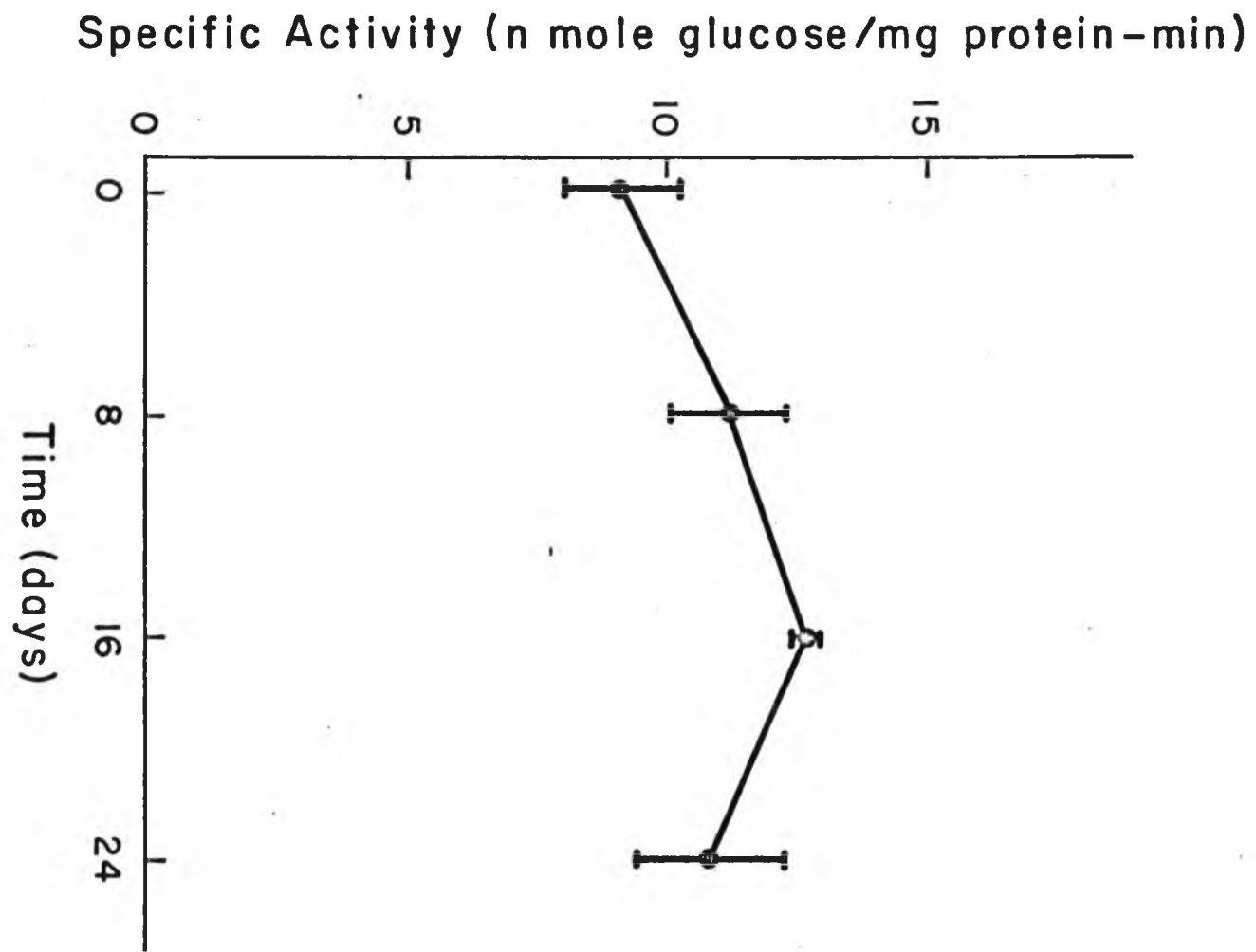
soluble invertase may play a greater role in sucrose degradation than sucrose-UDP glucosyltransferase in the endosperm culture system.

Phosphorylase

Phosphorylase activity in the endosperm cultures was studied following sucrose supplementation. The activity of phosphorylase was found to be directly proportional to the incubation time ($r = 0.995$). The rate of glucose transferred from G-1-P to amylopectin was constant during the first 3 hours and independent of G-1-P concentration (Appendix Figure 10). Phosphorylase activity could not be detected in this enzyme assay mixture without the presence of NaF and Hg_2Cl_2 . NaF and Hg_2Cl_2 were found to suppress the activity of acid phosphatase and β -amylase which might degrade the newly formed starch in the enzyme assay mixture (Whelan, 1955).

Phosphorylase activity was low in the endosperm cultures during starch synthesis. During the first 16 days, when starch was rapidly synthesized in the endosperm cultures, phosphorylase activity appeared to increase slightly and remained fairly constant to 24 days (Figure 4). In the developing endosperm of corn, Tsai and his coworkers reported that phosphorylases II and III were not detectable 8 to 12 days after pollination (Tsai et al. 1970), but their activity increased significantly as starch increased in later stages. Ozbun et al. (1973) reported that phosphorylase was found at all stages of corn kernel development. During the early stage, phosphorylase activity was low but increased 6- to 10-fold as starch increased 200- to 300-fold in the developing endosperm (22 days after pollination). It is possible that

Figure 4. Phosphorylase activity of endosperm cultures after sucrose supplementation.



in the endosperm cultures, the low starch content may be related to the low phosphorylase activity.

Starch granule bound ADP-glucose : starch glucosyltransferase,
ADP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase

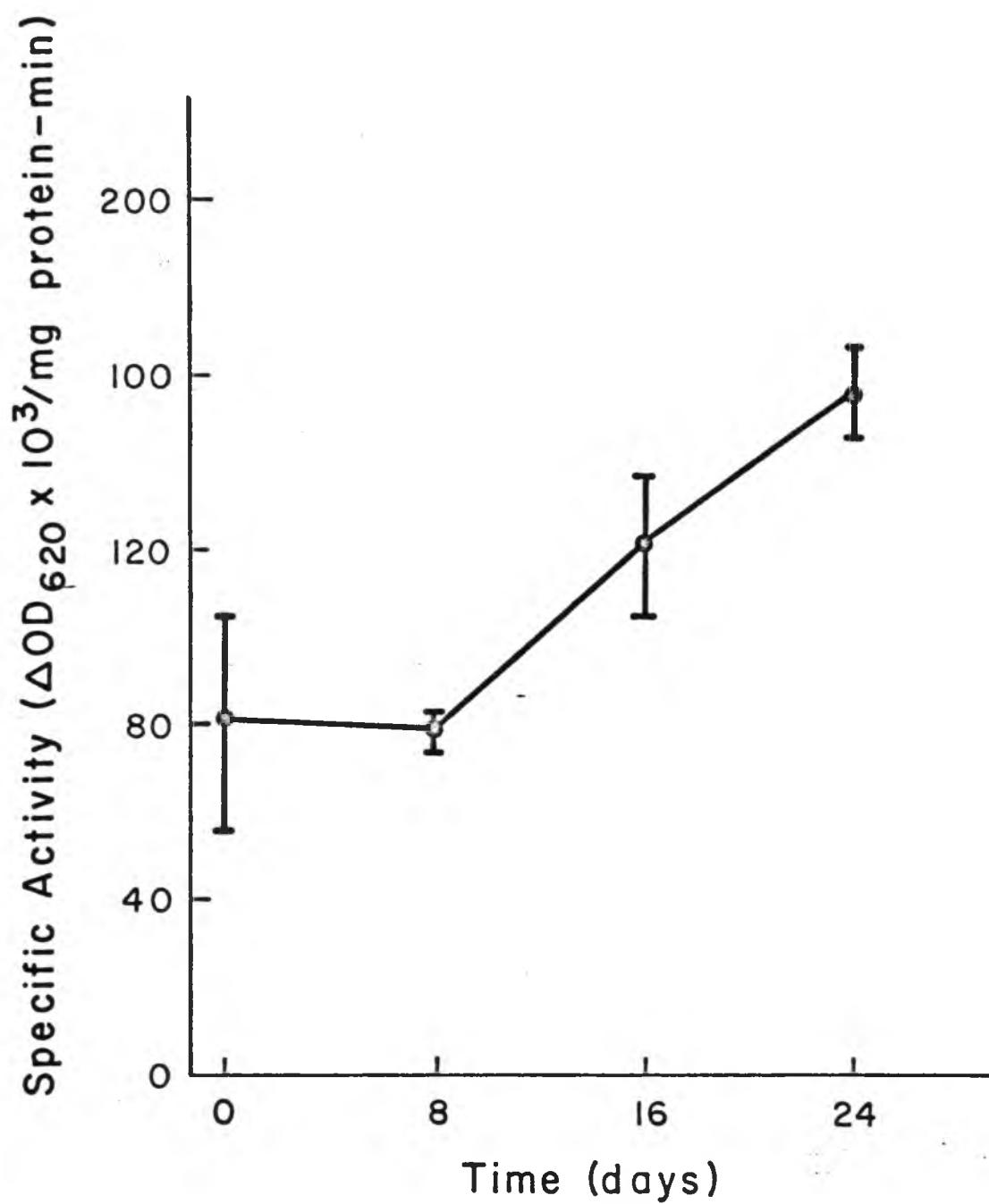
Activities of starch granule bound ADP-glucose : starch glucosyltransferase, ADP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase could not be detected using the procedures used by Tsai et al. (1970) and Ozbun et al. (1973). In the early developing corn endosperm, their activities have been reported to be absent or low (Tsai et al., 1970; Ozbun et al., 1973). The failure to detect the activities of these enzymes may be due to their absence or inadequate assay techniques.

Amylase

Amylolytic activity in the endosperm culture following sucrose supplementation was studied. The activity of amylase was directly proportional to the incubation time ($r = 0.995$) and the reaction rate was constant during the first two hours (Appendix Figure 11).

During the first eight days starch was formed rapidly in the endosperm culture and amylolytic activity remained constant (Figure 5). At this stage sucrose was probably adequate in the culture medium and additional energy obtained from degradation of starch was not required. Later, amylolytic activity in the cultured cells increased with time (Figure 5), which may indicate that most of the sucrose in the culture medium was consumed and degradation of starch as an energy source for the cultured cells became important.

Figure 5. Amylase activity of endosperm cultures after
sucrose supplementation.



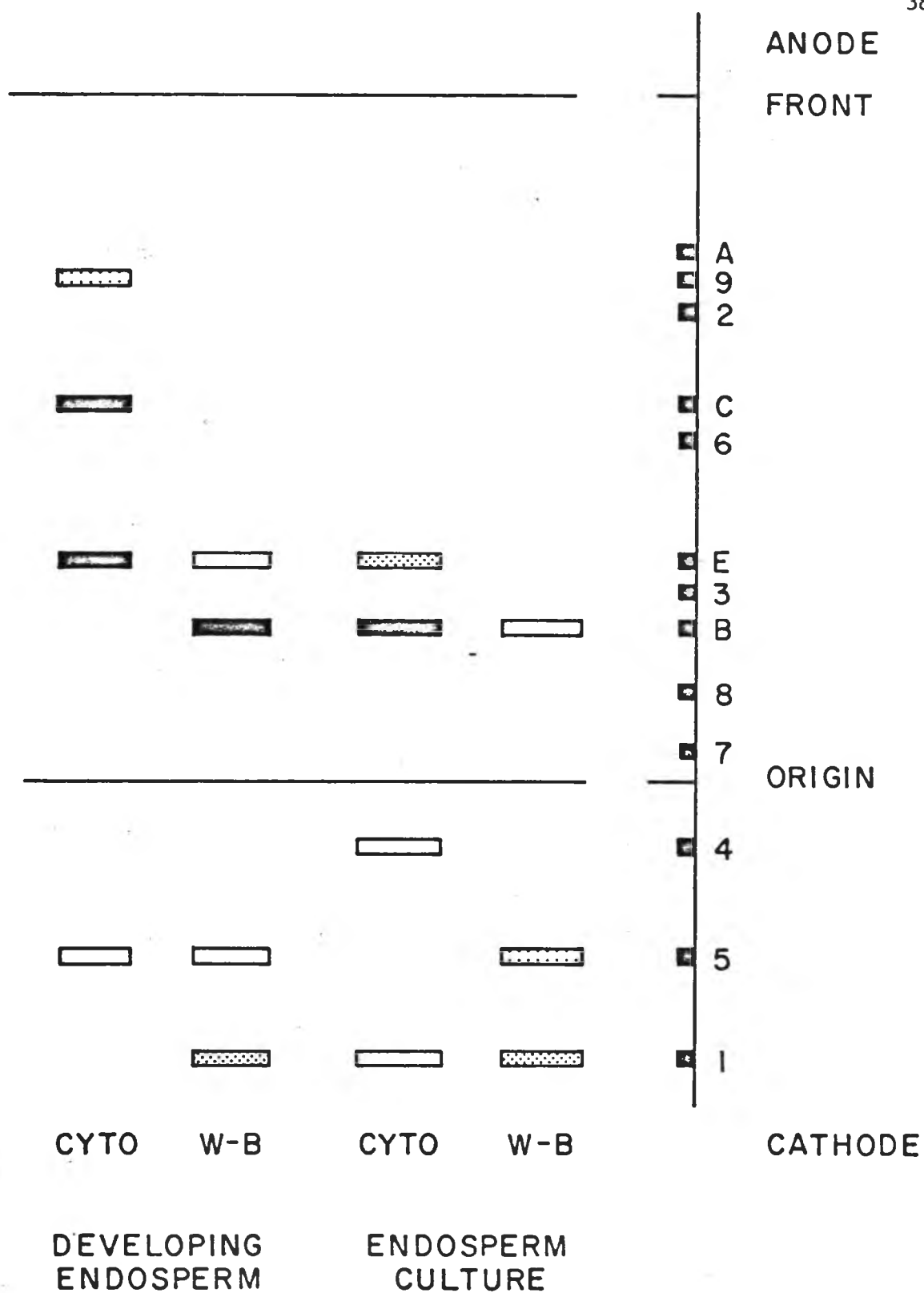
Peroxidase

The peroxidase isozymes in this study were designated according to Brewbaker and Hasegawa (1974). Figure 6 shows the zymogram of peroxidase isozymes in maize developing endosperm of inbred A636 (14 days after pollination) and endosperm cultures (16 days after sucrose supplementation). It includes both soluble (cytoplasmic) and cell wall-bound peroxidases of both tissues.

Among these peroxidase isozymes, there was one isozyme (E) which has not been established with certainty. This isozyme has a Rf value of 0.28 on the gel and was present in both soluble and cell wall fractions of developing endosperm and the soluble fraction of endosperm cultures. It was absent in the cell wall fraction of endosperm cultures. Although this isozyme was found in these three fractions, enzymatic and genetic evidence is needed to prove that it is the same isozyme.

Isozyme B was another peroxidase active in these tissues. This isozyme was reported to be present only in maize root and repressed in other tissues (Brewbaker and Hasegawa, 1974). It appeared as an active cytoplasmic enzyme in the endosperm cultures, but as a much less active enzyme in the cell wall fraction. In the developing maize endosperm, there was one isozyme found in the cell wall fraction which occupied a similar position as isozyme B. Since the observations that isozyme B was a cytoplasmic enzyme and isozyme 8 was found to be cell wall-bound in some tissues (Brewbaker and Hasegawa, 1974), it is possible that this is a fast band of isozyme 8. Further enzymatic and genetic studies are needed to confirm this suggestion.

Figure 6. Zymogram of peroxidases in soluble (Cyto) and cell wall-bound (W-B) fractions of 14-day old endosperm and endosperm cultures 16 days after sucrose supplementation.



Peroxidases 1 and 5 were found to have higher activities in cell wall-bound fractions in developing endosperm and endosperm cultures, confirming previous results (Brewbaker and Hasegawa, 1974).

There were other reports on the study of peroxidase in maize endosperm. Levites and Maletsky (1972) using vertical electrophoresis with starch gel, identified twenty-nine peroxidase isozymes in 16-day old corn endosperm. Forty-four inbred lines of maize were studied. Lodha and his coworkers reported seven peroxidase bands present in normal corn endosperm 15 days after pollination. They also found that the number of peroxidase bands decreased during grain development (Lodha et al., 1974). In this study, using the single inbred A636, there were four bands in soluble fraction and four in cell wall-bound fraction in the 14-day old endosperm. The different zymograms might be due to different genotypes, extraction methods, electrophoresis conditions or staining methods. More enzymatic and genetic studies are needed to provide a better understanding of the peroxidase isozymes in maize endosperm cultures.

No differences were observed in the polymorphism of peroxidase isozymes of 10-, 14- and 18-day old developing endosperm. In maize endosperm cultures, there was no difference in the peroxidase zymogram at 0, 8, 16 and 24 days after sucrose supplementation. However, zymograms of peroxidases in developing endosperm and endosperm cultures were different. With limited understanding of the physiological roles of each peroxidase, it is difficult to interpret these differences between the tissues.

SUMMARY

Activities of several enzymes which are involved in sucrose degradation (invertase, sucrose-UDP glucosyltransferase), starch formation (phosphorylase, starch synthase) and starch degradation (amylase) were determined in maize endosperm cultures following sucrose supplementation. Dry weight and starch content of the endosperm cultures increased following sucrose supplementation.

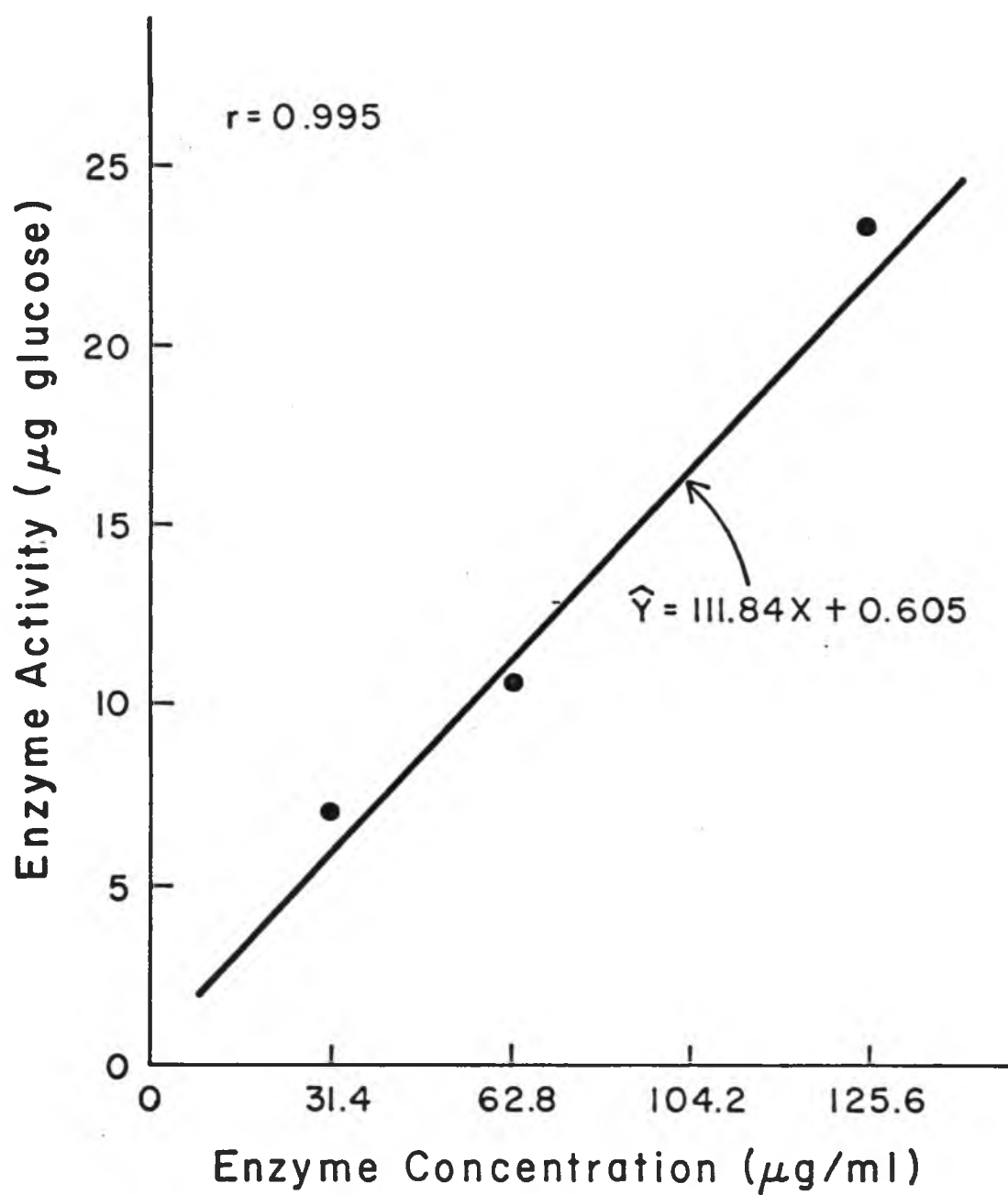
The specific activity of soluble invertase remained high and fairly constant, while cell wall-bound invertase decreased after sucrose supplementation. Compared to invertase, sucrose-UDP glucosyltransferase activity was low in the endosperm cultures. Thus, invertase may play a greater role in sucrose degradation than sucrose-UDP glucosyltransferase in this culture system.

Phosphorylase activity was low during starch synthesis in the cultured cells. Activities of starch synthase, ADP-glucose pyrophosphorylase and UDP-glucose pyrophosphorylase could not be detected due to their absence or improper assay techniques. Low starch content in endosperm cultures may be related to the activities of these starch forming enzymes.

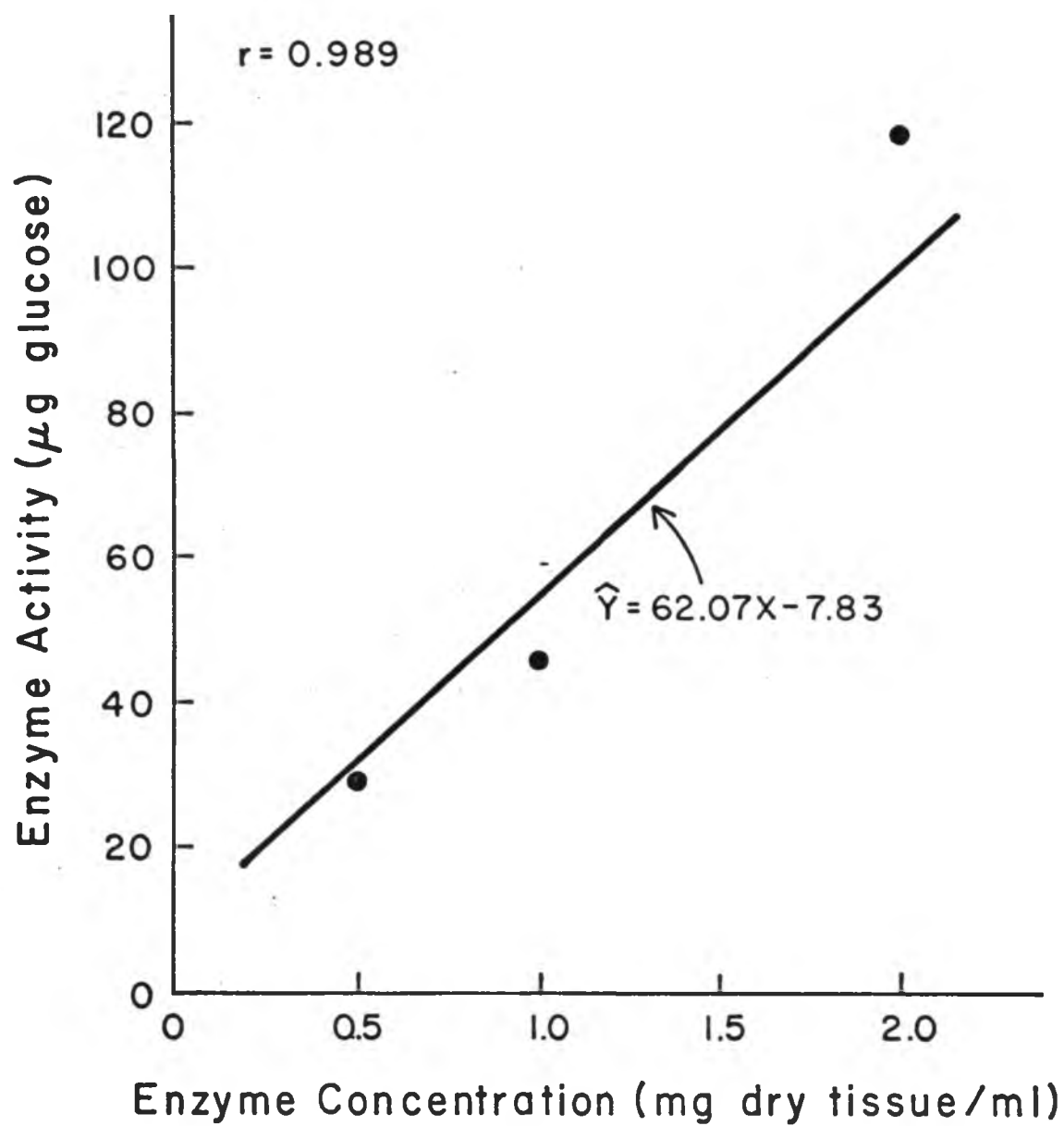
Amylase activity increased after eight days. This may indicate that most of the sucrose in the culture medium was consumed and energy obtained from starch degradation was required.

Zymograms of peroxidases in endosperm tissue cultures and developing endosperm were different. It is possible that the differences are due to the different growth environments. No ontogenetic variations were observed in the peroxidase isozymes in these two tissues.

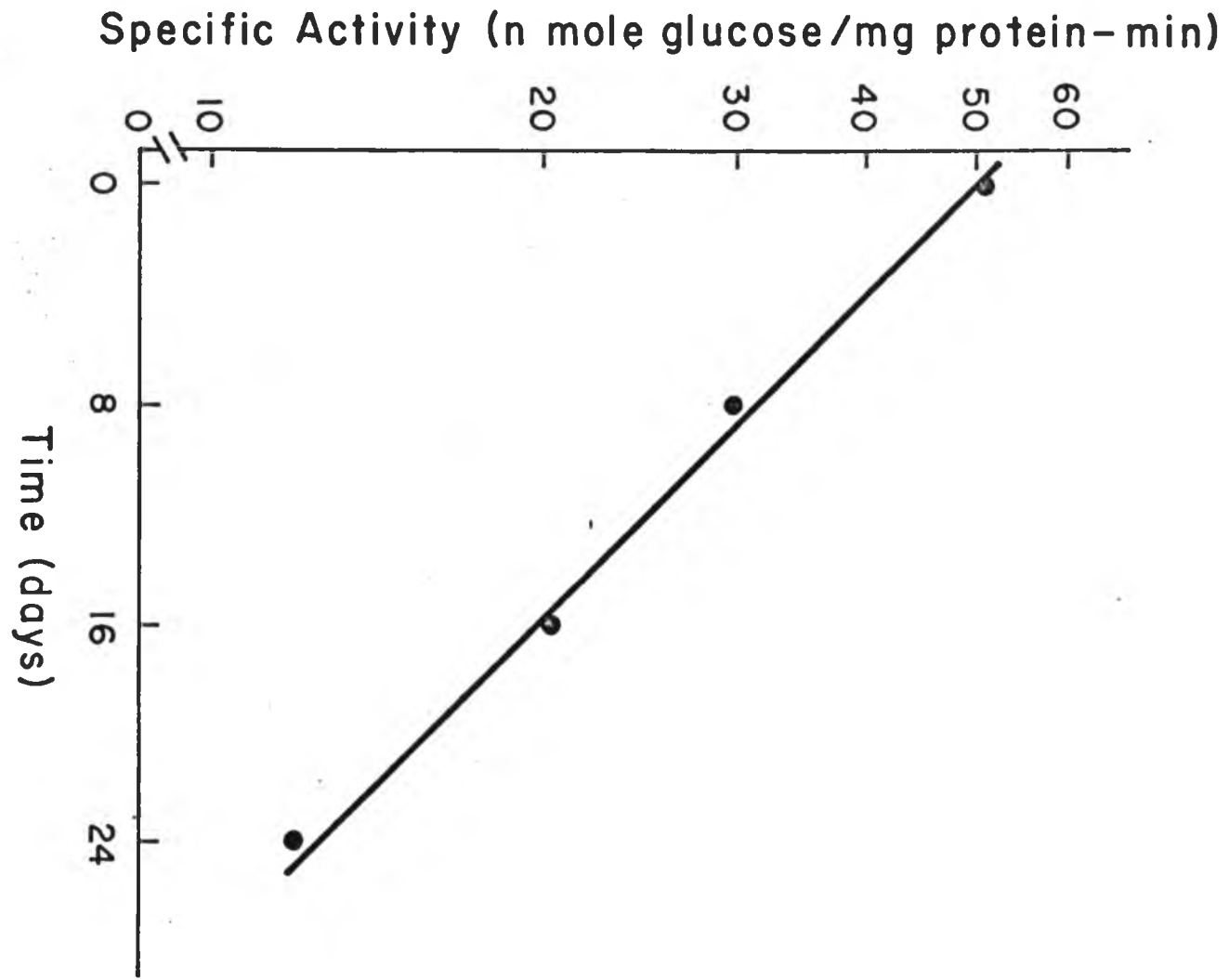
Appendix Figure 7. Soluble invertase activity of endosperm cultures (16 days after sucrose supplementation) as a function of enzyme concentration.



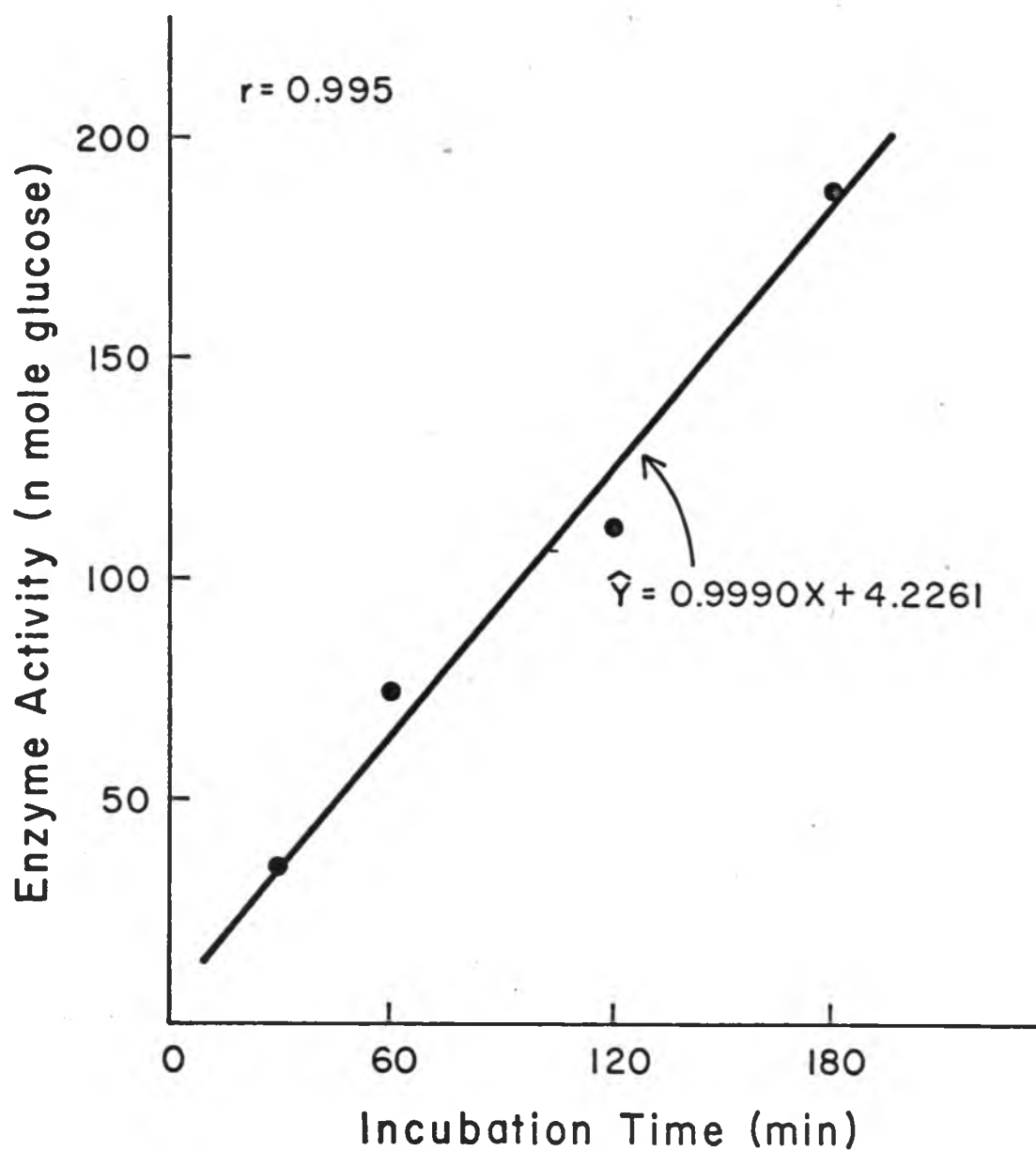
Appendix Figure 8. Cell wall-bound invertase activity of endosperm cultures (16 days after sucrose supplementation) as a function of enzyme concentration.



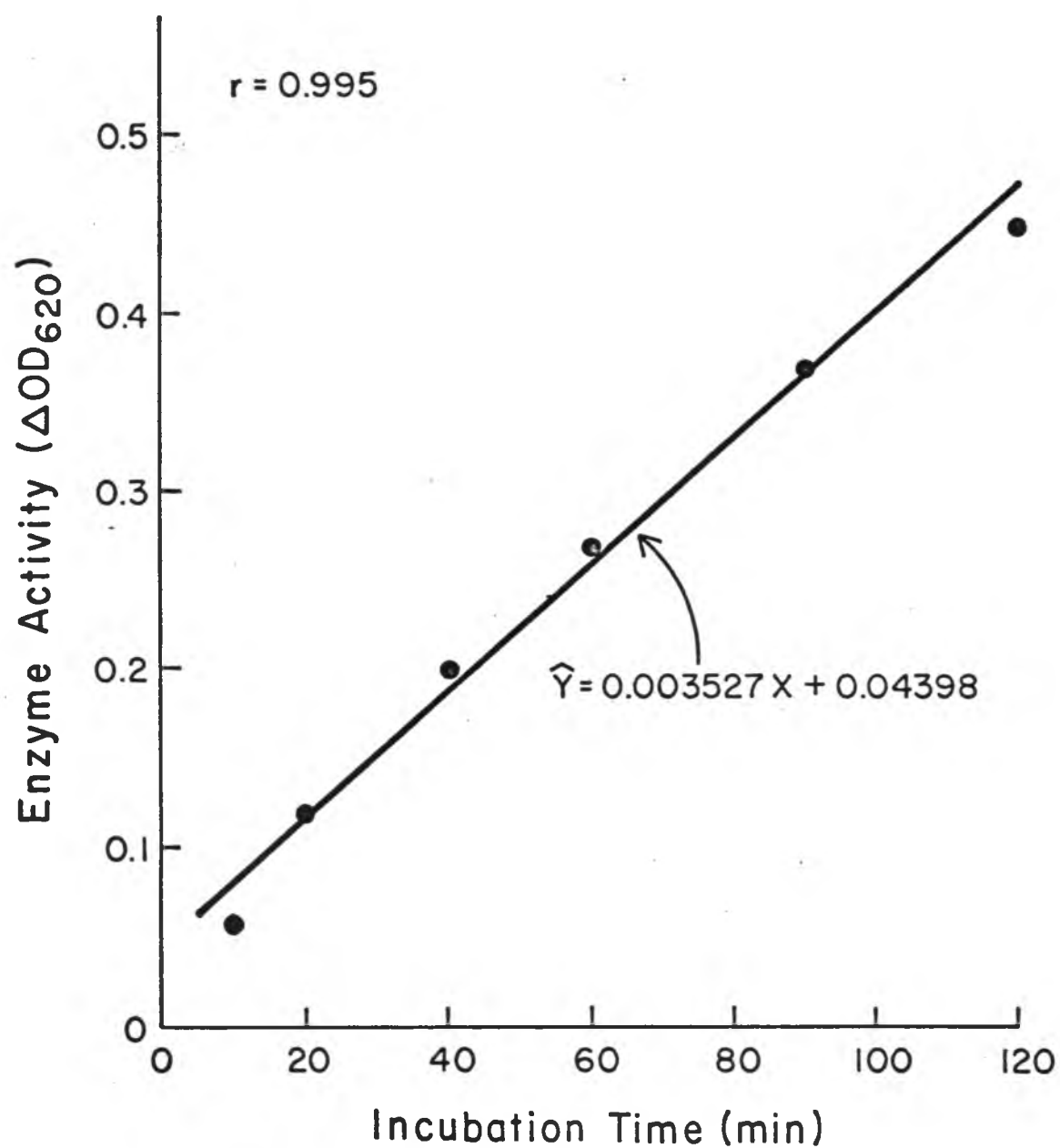
Appendix Figure 9. Cell wall-bound invertase activity of endosperm cultures as a log function of time after sucrose supplementation.



Appendix Figure 10. Phosphorylase activity of endosperm cultures (20 days after sucrose supplementation) as a function of incubation time.



Appendix Figure 11. Amylase activity of endosperm cultures
(20 days after sucrose supplementation) as a function
of incubation time.



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